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PATENT

PROVISIONAL APPLICATION FOR PATENT COVER SHEET
(37 CFR §1.51(c)(1))

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Title of Invention: **3-D ELEMENTAL COMPOSITION AND STRUCTURE OF BIOLOGICAL AND ORGANIC MATERIALS WITH ATOM PROBE MICROSCOPY**

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Provisional Patent Application:

3-D Elemental Composition And Structure Of Biological And Organic Materials With Atom Probe Microscopy

Confidential
Inventors - Steven L. Goodman and Thomas F. Kelly
Imago Scientific Instruments

August 3, 2003

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to methods and instrumentation to determine the three-dimensional structure and elemental composition of biological materials at the molecular, near atomic, and atomic level using 3-D atom probe microscopy and related methods. This invention is made practical by the development of improved 3-D atom probe microscopes, as exemplified by Imago Scientific Instruments' local electrode atom probe (LEAP). Such improvements include substantial increases in the microscope field of view, the geometry of the specimens that may be examined, and much more rapid analysis times. Nonetheless, the invention described herein can be applied to conventional atom probes by those skilled in the art, hence this invention also pertains to determining the structure of biological and organic materials with other types of atom probes and related instruments, where feasible.

The methods and instrumentation to enable the structural and compositional analysis of biological and organic materials, that is the subject of this invention, include proteins, lipids, carbohydrates, and nucleic acids, as well as biomolecular and biomacromolecular assemblies such as receptor complexes, receptors coupled with ligands, coupled enzyme-substrates, and coupled drugs with target biomolecules, membranes, membrane proteins, cellular organelles, cells, and other biological components including tissue specimens.

This invention also relates to methods and instrumentation to determine the three-dimensional structure and elemental composition of synthetic and natural organic materials at the molecular, near atomic, and atomic level. This includes certain organic materials, coatings and thin films, polymers, polymeric and organic composite materials, liquid crystals, and both synthetic and natural materials that may contain multiple organic materials in association with inorganic materials.

One particular area of application of the present invention is determining the three-dimensional structure and elemental composition of proteins and/or oligonucleotides for applications in proteomics, genomics and pharmaceutical research and development.

A second particular area of application is the determination of the nanostructure and elemental composition of proteins, macromolecular complexes or other structures that are located within

biological tissues, cellular components, cellular organelles, extracellular organelles, viruses, bacteria, other micro-organisms, or other biological systems or components.
A third particular area of application is the determination of the nanostructure and elemental composition of man-made or partially man-made biological structures including tissue engineering scaffolds, cell culture systems, and other biological-synthetic constructs.
A fourth particular area of application is the determination of the nanostructure of polymers, polymer coatings, and other coatings that may contain organic components.
A fifth particular area of application is the determination of the nanostructure of nano and micro scale biotechnological devices such as bio-array chips, biosensors, biomaterials, and bioMEMs.
A sixth particular area of application includes determination of the nanostructure of materials that may not be organic or biological, but that may be prepared and analyzed using means that are similar to those described herein. Such materials include fullerenes, ceramics, dielectrics, nano and micro-porous catalysts, zeolites, materials with nanoscale voids and cavities, colloids, and micro and nano-particulate materials.

2. Description of Related Art

Detailed understanding of the atomic and molecular structure of biological molecules including proteins, nucleic acids, lipids, polysaccharides, and other components is at the root of much contemporary biology, medicine, and biotechnology, since it is the 3-D atomic and molecular structure that ultimately determines biomolecular function. For example, the three-dimensional structure of proteins determines the activity of enzymes, the specificity of receptors and antibodies, and how proteins (and other biomolecules) interact with each other. Targeting drugs to specifically interact with certain proteins, and to particular ligands on these proteins, is similarly also dependant on the atomic and molecular structure of a protein. The structure of DNA and RNA is of similar importance in determining function, drug targeting, and understanding biological processes. Because of the importance of the three-dimensional structuré of a proteins and nucleic acids, there has been considerable development of methods to determine these structures. For a detailed understanding of structure-function relationships, near atomic structural resolution of a few angstroms (tenths of nanometers) is required. Current methods to approach this resolution with biomacromolecular and organic structures can be categorized as:

- Computational methods that determine protein structure based upon knowledge of the primary sequence of amino acids. Similar methods may also be applied to other organic materials.
- Methods which utilize the diffraction of x-rays through crystals of protein, nucleic acid, or other molecules, and closely related methods utilizing the diffraction of other electromagnetic waves and particles (electrons, neutrons).
- Methods that utilize nuclear magnetic resonance to determine the proximity of chemical moieties to each other.
- Methods based upon high-resolution microscopies, including those based upon electrons, scanning tunneling, and atomic force.

Each of these methods has its limitations as discussed below.

Calculation based methods: The major difficulty for computationally determining 3-D structures based upon knowledge of the primary amino acid sequence of a protein is that these

require massive calculations. A small protein of only 100 amino acid residues has an astronomical number of possible conformations, on the order of 10^{16} (see K. A. Dill, Biochemistry, 24, 1501-1509, 1985). Moreover, the chemical or mathematical rules to choose the proper conformation are not entirely known. In fact, since proteins exist in a dynamic equilibrium with water, salts, other proteins, and various other biomolecules, the biological conformation may not be the lowest energy state, but in some (unknown) higher state. Rather than calculate the conformation *de novo*, some computational methodologies utilize sequence homologies. With such methods, proteins which contain similar sequences and which have a known conformation are used as a basis to calculate the structure of the unknown protein. However, this assumes that the conformation of a certain protein portion will be the same in both the known and unknown protein. Such an assumption may not be generally correct. Secondly, this also requires that there are known structural homologies, and these structures therefore must be determined by some other means. Finally, computational methods for determining protein structure from amino acid sequences are at best only partially generalizable for determining the conformation of other biomolecules such as nucleotides, nucleoproteins, carbohydrates, glycosaminoglycans, and proteoglycans, or for non-biological organic materials such as synthetic polymers.

X-Ray Crystallography: Methods based upon X-ray crystallography regularly provide the highest resolution of current methods, on the order of 2-3 angstrom atomic level resolution for proteins, nucleic acids, synthetic polymers and many other materials. However, obtaining such information is a very slow process. The analysis of x-ray diffraction data can take months in order to determine structure since diffraction patterns are extremely complex, consisting of as many as 25,000 diffraction spots from a single small protein. Solving a diffraction pattern has been described as directly analogous to reconstructing the shape of rock from the ripples it creates when thrown into a pond (see: Molecular Cell Biology, Lodish et al, Scientific American Books, New York, 1995). Additional difficulties in crystallographic analysis arise because the molecule under study must be first crystallized into a highly regular crystal with millimeter or near millimeter dimensions. This therefore requires that the (bio)molecule under study must be removed from its normal milieu or produced in quantity by various means, be highly concentrated, and then crystallized. Such sample preparation alone can also take months. In addition, according to US patent #5597457: *System and method for forming synthetic protein crystals to determine the conformational structure by crystallography*, less than two percent of the over 100,000 known different proteins have been grown as crystals suitable for x-ray diffraction studies. Secondly, the resolution obtained is limited by the quality of the crystal. Due to the time required for crystallization and the collection and analysis of diffraction data, determining the structure of a single macromolecule can readily take one year or more. Finally, crystallization is considerably more difficult and not even be feasible with many important biomolecules such as certain membrane proteins, pharmacologically important receptors, and macromolecular complexes.

Nuclear Magnetic Resonance: High-resolution nuclear magnetic resonance (NMR) can provide angstrom-level resolution comparable to X-ray crystallography on many types of biological and organic samples. Like crystallography, NMR also requires separation and purification, hence the molecule of interest must be isolated from its normal environment thereby potentially altering its conformation. The higher resolution method of liquid state NMR (in

contrast to solid state NMR) is generally limited to molecules of a molecular weight no larger than about 40kD due to the requirement that the molecules rapidly tumble in solution. Some recent reports suggest that the latest generation high field NMR magnets operating at 800-900 MHz, may enable the determination of structures up to 100kD. Nonetheless, high resolution NMR requires that the biomolecule must be removed from its natural environment and then solubilized. In addition, protein samples for NMR are often isotopically labeled in order to facilitate the determination of the relative location of particular chemical moieties, thereby providing an additional manipulation that can alter structure. High resolution NMR is not able to analyze high molecular weight (MW) biomolecules, such as many larger proteins and macromolecular complexes. Although solid state NMR does not have the size and solubilization limitations of liquid state NMR, the lower resolution limitation of solid state NMR is only able to provide nanometer-level resolution, and not the necessary angstrom level resolution. Finally, the analysis of NMR spectra is complex, time consuming, and less than straightforward.

High Resolution Microscopy: Current scanning and transmission electron microscope technology is not able to provide the necessary sub-nanometer resolution with biological and organic materials. While high-resolution transmission electron microscopies (TEM, intermediate and high voltage transmission electron microscopy; IVEM and HVEM, and scanning transmission electron microscopy; STEM, and scanning electron microscopies; SEM) can provide instrumental resolution in the angstrom range with some robust materials (e.g.: metals and ceramics), this is not possible with organic materials since low atomic number organic elements provide low intrinsic contrast and are easily destroyed by electron beams. In practice, the obtainable resolution for most biological and organic materials is, at best, in the 1-5 nm range, and more often in the 5-10 nm range. This is 10-50 fold poorer than the required atomic level. Higher resolution electron imaging in the 1-3 nm range and potentially higher can in some cases be achieved by averaging multiple images of identical and ideally dimensionally regular structures such as certain macromolecular complexes or viruses. However, angstrom-level resolution is still not achieved. Moreover, such images as are obtained are that of an average structure, and consequently individual details may be averaged out. Finally, electron microscopy does not directly provide compositional or elemental information. These are generally provided by indirect radiative emission methods (EDX, WDS), and by other methods such as electron energy loss spectroscopy (EELS) and related techniques. With carbon and the other low atomic number elements found in biological and other organic materials, the obtainable spatial resolution for elemental mapping is rarely better than about 10 nanometers and provides limited sensitivity. Scanning probe microscopies, including AFM, STM, NSOM, and related methods, are also not suitable for providing 3-D atomic resolution and elemental mapping of biological and organic materials. While atomic spatial resolution is possible, this instrument class only probes sample surfaces, while providing no information on underlying layers. Secondly, scanning probe microscopies do not provide information on elemental composition.

Need: Consequently, there remains a need to readily determine the three-dimensional structure and composition of biological and organic materials with atomic and/or near atomic level resolution.

3. Description of the Invention

Overview and background to current invention

The vast majority of specimens previously analyzed using atom probes have been metals, due to the need for high electrical conductivity and physical strength with the required millimeter-long sharp needle-shaped specimens. Consequently, there is only a modest history of atom probe analysis of biological or organic specimens. Field ion (FIM) images of freeze-dried tRNA dimers adsorbed onto iridium tips were reported by Machlin in 1975 (Machlin, Freilich et al. 1975), while Panitz has provided what appears to be the most recent report of FIM and field desorption imaging, which was applied to ferritin (Panitz 1981; Panitz 1982) and unstained DNA (Panitz 1983). Other than a few additional publications by these research groups, this is essentially the entire history of biological atom probe and FIM imaging. A major problem discussed in some detail by Machlin et al., is that the high field strengths for FIM imaging (~5-15 V/nm) appeared to cause desorption and fragmentation of adsorbed biomolecules. (Machlin, Freilich et al. 1975). While Machlin et al. saw macromolecular desorption or fragmentation as a problem, this can in fact be an opportunity. By accelerating the ionized macromolecular fragments to the two-dimensional microchannel plate detector, both positional and molecular weight information may then be obtained. Therefore, atom probes can then, in principal, be used to determine both 3-D structure and atomic or molecular composition. Such determination would greatly benefit structural biology, biomedical science, biomolecular engineering and related disciplines.

There have also been several reports that utilized atom probes to examine non-biological organic materials. Nishikawa examined diamond-like carbons with a non-imaging (one dimensional) atom probe to obtain compositional depth profiles (Nishikawa, Sekine et al. 1997; Nishikawa, Sekine et al. 1998). Other investigators used FIM and atom probes to examine graphite fibers (Liu and Tsong 1988), the intrinsically conductive polymer (ICP) polypyrrole (Maruyama, Hasegawa et al. 1987), and phthalocyanine (Iwatsu, Morikawa et al. 1987).

Although this history is limited, these reports indicate the potential to use atom probes to analyze conductive carbon-based materials. However, the masses of ionized fragments in these reports were considerably larger than one or a few atoms, but rather were generally rather large molecular fragments of 5-8 and or more atoms in size exclusive of hydrogen. Moreover, many ions were small polymers consisting of 2 or more repeat units. Such large fragment and small polymers are not desirable for determining the structure of macromolecules with an atom probe since the size of an ionized fragments determines spatial resolution, hence the spatial resolution is decreased with large ionized molecular fragments. In addition, determining the composition of larger fragments requires higher mass resolution. Therefore, for ideal atom probe determination of biomolecular structures, the ionized fragments will be individual atoms or small molecular fragments, preferably on the order of 2 to 4 atoms exclusive of hydrogen. Larger ionized molecular fragments that have a known and determinable 3-D conformation, and that are small in size, will not significantly decrease the use of the atom probe for structural analysis of macromolecules. Examples of such moieties include phenyls, styrenes, and other ring structures, and many pendant R-groups such as sulfonates and methyls.

Due to advances in atom probe instrumentation, especially as exemplified by Imago's LEAP microscope (Kelly, Camus et al. 1995; Kelly and Larson 2000), atom probe imaging of biological and organic materials (and many other materials as previously addressed) becomes practical. These advances include the LEAP's much larger field of view of circa 100 nm (or larger), which is substantially larger than conventional atom probes that generally provide a field of view less than 20 nm. Since biomolecules and macromolecular complexes are typically 10 to 100 nm or larger, a 10-20 nm field of view is generally inadequate. Secondly, the LEAP provides much faster data acquisition speed of ~10,000 ions/second, in comparison to circa 10 to 100 ions/second in comparison to other atom probe designs. This additional speed greatly accelerates analyses that might have required days or weeks with other atom probes to now be achieved in minutes or hours with the LEAP. Finally, the LEAP's local electrode design provides substantial benefits with respect to the geometry of specimens that may be analyzed. Specimens need not be circa 5 mm long needles that in turn must be mechanically sturdy and have a high level of electrical conductivity. Since the specimens may be only a few tens of microns in length, this enables less sturdy materials such as polymers to be analyzed, and since the conductive path is much shorter, lower conductivity materials may be analyzed. It should be recognized that the processes, methods and instrumentation described herein while explicitly developed for LEAP analysis, are also generally applicable for the preparation of specimens for other atom probes and related instruments.

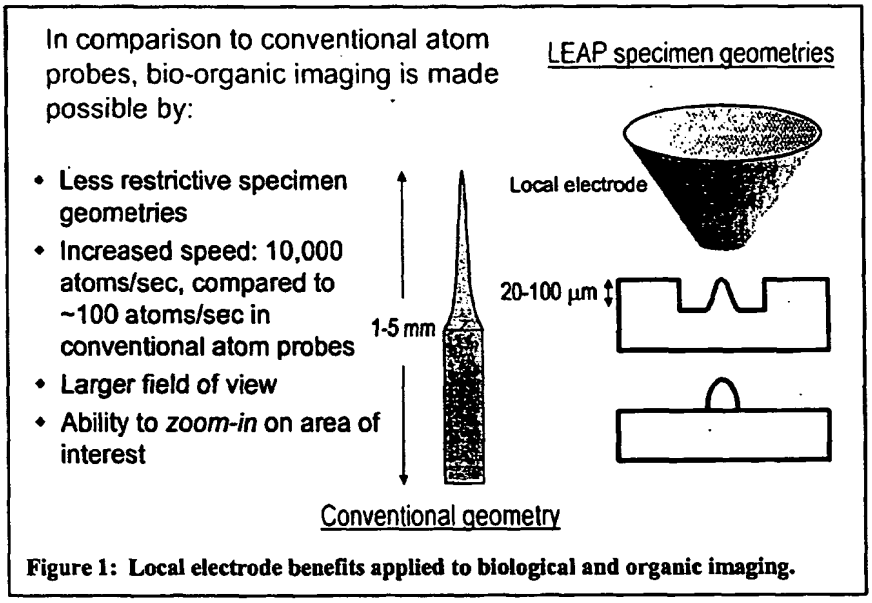

Figure 1: Local electrode benefits applied to biological and organic imaging.

Sample preparation

Overview: The LEAP and other atom probes are high vacuum instruments. Therefore, specimens that are normally hydrated, such as biological cells or tissues (that are on the order of 70% water by weight) must be carefully prepared in order to prevent rapid dehydration and collapse in the vacuum. Many of the sample preparation methods used for other high vacuum analytical instruments, such as electron microscopy, provide a starting point for such preparation. However, the special attributes and capabilities for atom probe and LEAP analysis require some different considerations, as discussed below for different types of organic and biological specimens. Sample preparation protocols can be separated into those that do not require

freezing, and those that utilize rapid freezing (cryogenic protocols). In contrast, many specimens that are not hydrated, or otherwise solvated, may be prepared without procedures to remove water (or solvent).

To examine biological materials with the LEAP (or other atom probes), several conditions must be met with respect to specimen preparation. These are discussed in Table 1.

Table 1: Specimen preparation conditions for atom probe analysis of biological and organic materials:

1. Native structure must be maintained in the vacuum environment prior to and during analysis.
2. Biological materials must be immobilized and stable in the high electric field of the atom probe (circa 3-15 V/nm).
3. Biological materials must field ionize from the surface of the specimen in a controlled manner as single atoms or small molecular fragments.
4. The specimen must be prepared with a needle-like geometry wherein the end of the needle must have a radius of no more than a few hundred nanometers.
5. The end of the needle must form a uniformly curved and smooth surface in the electric field, and this must be maintained during analysis.
6. The material must have a sufficient level of electrical conductivity.

The conditions discussed in Table 1 may be addressed as follows:

Condition 1- Maintain native structure:

Maintaining native structure can be achieved by using methods similar to those for preserving native (or near native) structures for transmission electron microscopy (TEM) and/or scanning electron microscopy (SEM) analysis. It is important to recognize that TEM specimen preparation methods are able to provide near atomic resolution structure of biological materials (Baker, Olson et al. 1999; Baumeister and Steven 2000; van Heel, Gowen et al. 2000). In fact, the primary limitation for EM resolution of biologicals is not specimen preparation but is due to destructive interactions of the electron beam with a specimen. In TEM this is generally dealt with by averaging dozens of low-electron-flux low-contrast images. With LEAP, the energy density is actually less than that in TEM, and moreover controlled destruction of the specimen one atom at a time is the goal. In other words, specimen damage in the LEAP is not as significant an issue since the LEAP does not image the same specimen region for a long period of time in order to statistically measure electron scattering. Thus, LEAP should be able to obtain atomic level resolution of organics that is superior to TEM biological resolution. (In addition, TEM does not provide elemental identification of low atomic number elements, does not provide any elemental identification of single atoms, and does not provide true 3-D imaging, as does the LEAP.) For LEAP specimen preparation, biological structure is maintained by removing the specimen water and replacing it with a resin or polymer with a low vapor pressure, similar to that done for TEM or SEM to preserve structure in the vacuum. A secondary approach is to freeze the water in place and to maintain this frozen state during subsequent preparative steps and while imaging. A third approach is to stabilize structure by embedding in non-organic materials.

Condition 2 – Stable in High Electric Field:

This condition, as well as condition 3, relate to the special needs for atom probe specimens to be stable in an electric field on the order of 10 V/nm. Briefly, biological materials must possess sufficient internal bonding stability so that they do not unfold or loose conformation in this high electric field, and that they do not simply "fly off" as whole molecules or fragments. This may be achieved by embedding the material in a supporting matrix, as described for Condition 1. Additional stability can also be achieved, as required, by introducing covalent cross-links within the specimen by reaction with suitable reagents such as aldehydes, dialdehydes, osmium tetraoxide, ruthenium tetraoxide, benzophenones, and other cross-linking chemistries. Other strong bonds, such as metallic and ionic bonds may also be used to stabilize the structure during analysis. With some specimen preparations, there may also be a need to introduce cross-links between the specimen and a supporting substrate.

Condition 3 – Field ionization from specimen surface as atoms or small molecular ions:
This condition also relates to the special needs for atom probe analysis of materials. In order to achieve high spatial and resolvable molecular identification with a given mass spectral resolution, it is necessary that individual specimen atoms or small molecular fragments should ionize, rather than large molecular clusters. Moreover, ionization must be controllable such that is occurring only due to voltage pulses (or due to other ionizing radiation pulses), and it must only occur from the top atomic layer of the specimen. With respect to ion size, smaller is better to achieve high spatial resolution and facilitate elemental identification. Ideally such ions should be single atoms or small molecular fragments. It is less desirable, but still acceptable, for ions to be small molecular fragments (generally less than 4 atoms exclusive of hydrogen). It is not very desirable to ionize large fragments (generally greater than ~10 atoms. Any ion larger than the

In an Atom Probe: Ionization Probability α ($\Sigma_{\text{bond energies}}$, geometry)

Material bonding	Bond energy (kcal/mol)	Atom probe analysis?
Metallic bonds	27-83	Extensive
Covalent bonds	15-170	Si, C (diamonds)
Ionic bonds	140-250	Rutile (TiO_2)
Hydrogen bonds	1-12	?
van der Waals	1-10	?

Metals and microelectronics: metallic, covalent bonds
Biotechnology materials: covalent, ionic, dispersion, hydrogen bonds

Table 2: Ionization probability in atom probes and types of bonds found in different materials. The third column indicates the extent of experience and types of materials with different bond types that have been examined with atom probe microscopes.

spatial resolution of the LEAP will degrade spatial resolution. (Spatial resolution in the LEAP is nominally 0.5 nm laterally and 0.2 nm axially. However, this is also a function of the geometry of the specimen and other specimen and instrument factors.) The ion size may be controlled by specimen preparation and other means.
Briefly, the ionization voltage for a given specimen atom is proportional to the bond strength of that atom within the specimen, and may also be influenced by the specimen conductivity and geometry. The ion mass or size (the number of atoms in a molecular cluster) may be controlled, by varying the bond strength within the sample. This is done by introducing strong bonds within the specimen and/or by varying the level of cross-linking within and to an embedment material. In most cases appropriate bonds are primarily covalent, but may also be ionic, or metallic as these are also high strength bonds. Weak bonds, such as hydrogen bonds and dispersion or dispersion bonds (van der Waals bonds) will be generally insufficient unless there are multiple such bonds. This is achieved in practice by treating the specimen with cross-linking agents such as glutaraldehyde and osmium tetraoxide, as well as other agents. In general, the more highly cross-linked a specimen is, the smaller the fragments that will be ionized since each atom or R-group will be more tightly bound to its neighbors. This will ensure that the bond(s) that will break upon field ionization will be weakest bond in the highest field zone, which will be the bond that is closest to the local electrode in the LEAP, or to the remote electrode in a conventional atom probe. Thus, the surface atoms will preferentially field evaporate, and not atoms below the specimen surface.

Condition 4 - Needle-like specimen geometry:
The specimen must have a needle-like geometry in order to focus the high electric field of several V/nm at the specimen surface. With conventional atom probes the needle must be several millimeters long. With the local electrode atom probes such as the LEAP, this needle may be much shorter, on the order of a few tens of microns, since the local electrode applies the field only in vicinity of the specimen tip (see Figure 1). With the local electrode the needle need be about as tall as the aperture of the local electrode. Few biological materials are sufficiently sturdy to maintain even a 10-100 um-long needle shape with a circa 100 nm radius tip, especially after water is removed. However, replacing specimen water with an embedding material of sufficient physical strength can provide the necessary mechanical support.

Condition 5 - Uniformly curved and smooth specimen tip:
Imaging atom probes are projection microscopes where the specimen is the lens. Minimal aberration is achieved when the lens, that is the tip of the needle-shaped specimen, is a smooth hemispherical surface. Although at the atomic scale it is impossible for the surface to be entirely smooth since each atom has a finite size, roughness larger than the size of individual atoms will introduce field irregularities that will convolve the image resolution. To achieve a smooth surface the entire specimen must uniformly ionize one atomic layer at a time. This, in turn, is realized by ensuring that there are no large variations in the bond strengths of the material that would cause weakly bound atoms (or small molecules) to field ionize from a location that is not at the specimen surface. In practice, this is achieved by stabilizing the internal bonds of the sample, and by having an embedding or supporting media that field evaporates small ions within the same field strength range as the embedded sample that is to be analyzed.

Condition 6 - Sufficient level of electrical conductivity:

The specimen must have sufficient level of electrical conductivity in order to concentrate the electric field at the specimen apex, and in order to conduct electrons from the tip as atoms or small molecular fragments field evaporate as positive ions (as in the LEAP). Since in one embodiment, the LEAP uses nanosecond duration electric pulses to induce field evaporation of specimen ions, the specimen must have sufficiently low impedance to rapidly conduct this charge to and from the specimen tip. With longer duration pulses the need for low impedance specimen preparations correspondingly decreases. When the pulsed ionization field used to induce single atom or small molecular fragments to ionize from the specimen surface is non-electric (such as photonic), insulating specimens may be analyzed.

In summary, most of these necessary conditions may be achieved by removing the specimen water and replacing it with an embedding material that imparts stability in vacuum, stability in the electric field, preserves structure, enables ionization of small molecular fragments and/or single atoms, and provides adequate electrical conductivity. This is diagrammatically shown in Figure 2. Additional properties required for embedding materials are indicated in Table 3.

STABILIZING SPECIMEN FOR ELECTRIC FIELD.

Technical Approach

- Strongly attach to substrate if required:
  - Au-thiols, Silanes, ...
- Promote ionization of single atoms or small molecular fragments
  - Introduce covalent cross-links as required
- Preserve structure in vacuum
- Support structure as atoms are ionized and removed
- Provide conductivity
  - Embed in conductive matrix

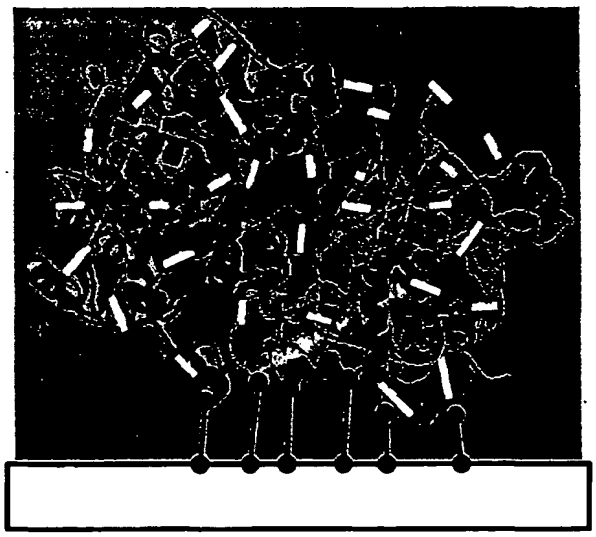

Figure 2: Diagram of a stabilized protein specimen prepared for atom probe analysis. Covalent cross-links are shown as white lines within ribbon protein structure. Black lines indicate thiol, silane or other bonds to link the protein to the substrate. Background indicates embedding matrix.

Table 3: Additional properties for embedding matrix materials.

- Freely diffusible into specimens to ensure good infiltration
- Minimal disturbance of structure during infiltration and polymerization or cross-linking or other processes to support embedded specimens
- Low out-gassing once in solid state, at least at cryogenic temperatures generally used in atom probe microscopes.
- Bond energy in same range as biological molecules. In most cases this is achieved with mostly covalent and/or ionic bonds as are common in organic materials.
- Discernable atomic structure from proteins, lipids, and other biological and organic materials that are to be encapsulated and examined with the LEAP. Since matrices will often be organic in composition, different embedding materials will be best suited for different specimens in order to enable discrimination of the embedment from the specimen.
- Uniform density and small domain size, on the order of a few nanometers, in order to provide structural uniformity.
- For rapid nanosecond voltage pulsing LEAP analysis the material will require semiconductor levels of electrical conductivity in the kHz range. Any necessary conductivity must occur with a sufficient level of spatial uniformity within the matrix.
- Specimens are generally imaged at cryogenic temperature during LEAP analysis. For polymers this would be at least below the polymer glass transition temperatures. Therefore, when conductivity is required the embedding matrix must provide this at such temperatures, which will typically be as low as 173 K (a very low polymer glass transition temperature). Maintaining conductivity at even lower temperatures is

In addition to removing specimen water and replacing it with an embedding matrix, it is also possible that the water can instead be frozen in place. It is also possible to combine these approaches and freeze the water while also embedding the specimen with a hydrogel or other polymer. This polymer or macromer can enhance mechanical stability, potentially provide some electrical conductivity (depending upon the polymer or macromer and any staining or other treatment), and provide chemical bonds to the water and to the specimen to provide for uniform ionization of the embedded specimen and embedding matrix. When the specimen and/or the embedding matrix includes frozen water then the atom probe must enable cryogenic transfer so that a frozen specimen can be introduced into the vacuum and will be maintained frozen continuously until analysis is complete.

Specimen preparation to achieve atom probe analysis of biologicals and organics

Intrinsically conductive polymeric embedding materials

Intrinsically conductive polymers (ICP) provide a nearly ideal combination of properties for embedding biological materials; namely adequate electrical conductivity and organic polymer composition hence having similar intramolecular organic bonds as found in biological specimens. The ability to successfully atom probe an ICP has been reported for polypyrrole (Maruyama, Hasegawa et al. 1987). Several ICPs have properties that make them useful for

embedding atom probe specimens. These include polyaniline, with a reported conductivity as high as 10 S/cm (Panipol F and T, from Panipol Ltd, Porvoo, Finland) and various polythiophenes, with conductivity as high as 30 S/cm (Baytron M from H.C. Starck, Inc., Pittsburgh, PA). These ICPs have physical properties that make them adaptable for the embedding biological tissues, proteins, cells, macromolecular complexes, biotechnology materials and devices, particulates, porous materials, and other materials as described elsewhere. These material properties include:

- Low viscosity, especially as monomers.
- Reasonable viscosity as prepolymers and polymers.
- Panipols and Baytrons can be blended with other resins and cross-linkers, to alter or optimize embedding properties, since conductivity is maintained in polymer blends.
- Baytron polythiophene is suitable for freeze substitution methods of specimen preparation since it can be polymerized from monomeric precursors; 3,4-ethylenedioxythiophene and Fe(III) toluenesulfonate (Baytron M + C) at low temperatures within organic solvents such as toluene and alcohols that are routinely used for TEM specimen preparation. This material also has suitable properties for non-cryogenic embedding protocols.
- Aqueous dispersion Panipols and Baytrons have reasonably low viscosity, hence many biological materials may be directly embedded from water, therefore requiring no initial dehydration. This is a novel property for an embedding matrix, as such direct aqueous embedding is not generally used in TEM. This type of embedding can simplify protocols and provide adequate structural preservation for certain applications.
- There are additional intrinsically conductive polymers that also have suitable properties or can be formulated to have such properties as listed in Tables 1 and 2.

These embedment materials can also be used for embedding non-biological materials for atom probe analysis. These include certain types of polymeric devices, drugs, liposomes, organic-inorganic composites, nano-biotechnological devices such as bio-array chips, biosensors, biomaterials, and bioMEMs devices, and many different types of nanoparticles and fragments of structures that are require mechanical support, void filling, providing a uniform ionization surface, electrical conductivity and other properties provided by the matrix. Some additional materials that can benefit from embedding include fullerenes, ceramics, quantum dots, dielectrics, nano and micro-porous catalysts, zeolites, materials with nanoscale voids and cavities, and other types of micro and nano-particulate materials not explicitly listed.

These ICP properties enable biological and certain organic specimens to be prepared using protocols that are somewhat similar to those used for the preparation of TEM specimens to preserve specimen structure. Certain of these formulations will enable embedding from organic solvents, embedding from water or water-miscible solvents, and may enable high-resolution cryogenic immuno-cytochemical protocols using freeze substitution (Table 3).

In addition to intrinsically conductive polymers or ICPs, polymers that can be made conductive via extrinsic treatment may also be used to support and embed specimens and fill voids. Such materials may have a significant fraction of unsaturated double bonds or other moieties that can be subsequently reacted with OsO_4 or RuO_4 to induce sufficient conductivity. Such materials include low MW dienes with hydroxyl groups and other water-soluble moieties, polymerizable diene monomers, certain unsaturated acrylics and epoxies, and other polymers or prepolymers

that be polymerized by free radical activation, photo-activation, or other means. These may then be cross-linked by OsO_4 or RuO_4 to provide further stability and some electrical conductivity. Of course, when an atom probe that does not rely on nanosecond pulsing to achieve ionization is used, then even lower conductivity polymers may be used to embed and stabilize biological specimens.

The overall approach to specimen preparation and LEAP imaging is diagrammed in Figure 3.

Protocols	ICP Name	ICP Chemistry	Solvents	Somewhat like known TEM resins
Organic solvent embedding En bloc and on tips	Panipol F Panipol T Baytron M + C	Polyaniline solution Polyaniline solution Polythiophene monomers*	Toluene Toluene Alcohols Acetone	Spurrs, Epon, Other epoxies, Some acrylics
Aqueous embedding En bloc and on tips	Panipol W Baytron P Baytron CPUD2	Polythiophene dispersion Polythiophene dispersion Polyaniline dispersion blend	Water	Lowicryl K4M, LR White
Freeze-substitution En bloc and on tips	Baytron M + C	Polythiophene monomers*	Alcohols Acetone	Lowicryl HM20

Table 3: Intrinsically conductive polymers (ICP) for embedding biological or organic specimens. *The polythiophene monomers are 3,4-ethylenedioxythiophene and Fe(III) toluenesulfonate. Panipol is available from Panipol Ltd., Baytrons from H.C. Starck, Inc., and polypyrrole from Aldrich (St. Louis, MO).

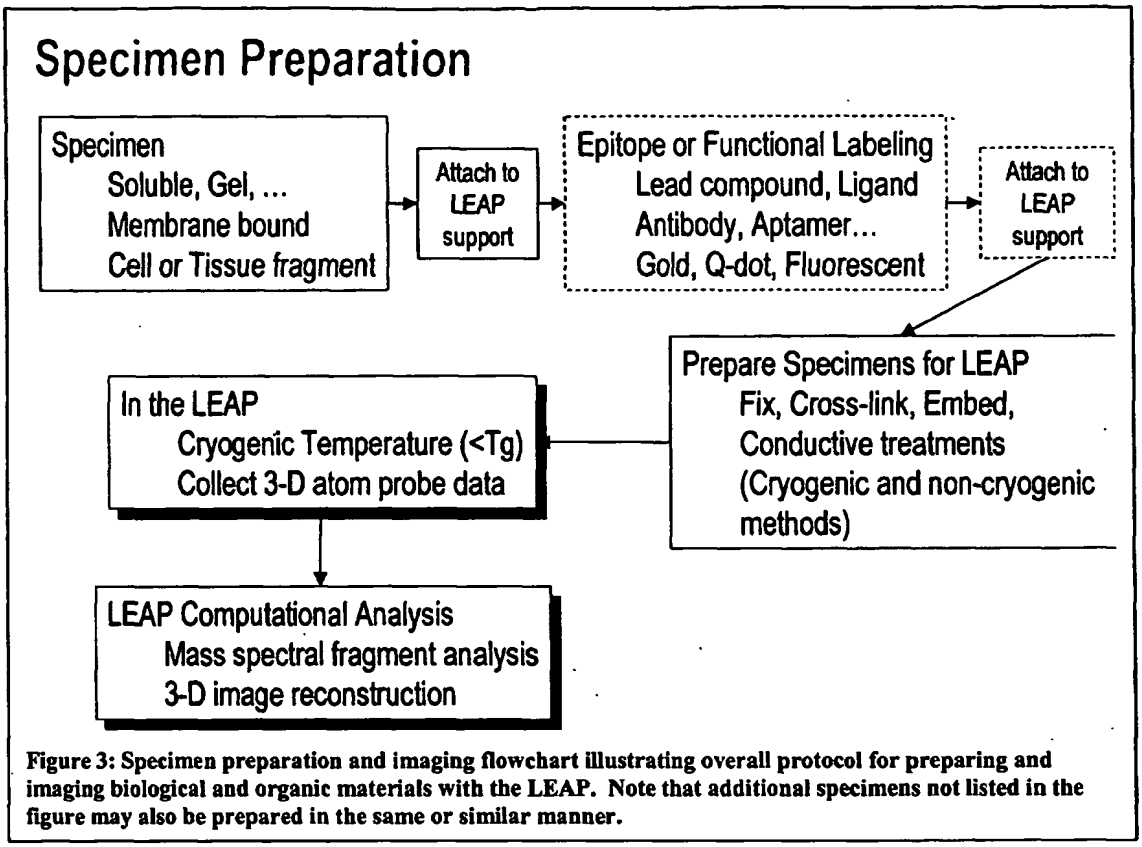

Figure 3: Specimen preparation and imaging flowchart illustrating overall protocol for preparing and imaging biological and organic materials with the LEAP. Note that additional specimens not listed in the figure may also be prepared in the same or similar manner.

Increasing the conductivity of ICP embedding materials

Since OsO_4 fixation provides strong cross-links to stabilize specimens, and can provide some conductivity and staining of specimens, the effects of OsO_4 treatment on the resistance of Baytron CPUD2 polythiophene was examined. Interestingly, we determined that the resistance of cast Baytron CPUD2 (two-point measure) decreased from 10 to 50-fold with 10-60 minutes exposure to dry saturated OsO_4 vapor in a closed vial. Cast Baytron P polythiophene also decreased its resistance to about ¼ of its initial value in a similar time frame. It was confirmed by removing the exposed surface of the Baytrons and measuring resistances again, that the decreased resistance was within the bulk material, hence was not a function of a surface coating of osmium. This was further confirmed by examining cross sections of the OsO_4-stained Baytrons with high-resolution secondary and back-scattered SEM. These observations indicated that the OsO_4 had penetrated into the bulk of the material; hence this was not a surface deposition effect. This demonstrates that osmicated polythiophenes have good conductivity properties for embedding biologicals. This also demonstrates a method to significantly increase the electrical conductivity of polythiophenes that may be useful for other applications of conductive polymers.

Since atom probe analysis is routinely performed at cryogenic temperatures, it is critical to determine how such temperatures affect electrical conductivity. The resistivity of non-osmicated Baytron P and CPUD2 specimens were assessed by continuous monitoring of DC resistance with a suitable as the temperature was changed in a LN2 cooled cryostat. It was observed that

CPUD2 increased its resistivity no more than two-fold (doubled) as the temperature was decreased from 298K to 97K. Baytron P increased its resistivity also by 2-fold over the same temperature range. 97K is well below the glass transition temperatures for polymers and organics, which are generally no lower than 120-170K. Such a doubling of resistance is minimal and thus should not have adverse effects on the use of these materials in embedment formulations and where specimens are examined at cryogenic temperatures.

Specimen preparation benefits of osmium treatment

The osmication treatment also improved the physical properties of Baytron P and CPUD2, as well as PANI F, for preparation of atom probe specimens. These materials were made substantially more rigid and tougher, hence were more easily cut to the proper shape and were more readily sharpened with an ion beam into the proper needle-shape geometry. An additional benefit of OsO_4 treatment is that osmium provides a high mass element for mass/charge calibration. While simple OsO_4 vapor exposure was used to treat these materials, additional methods can also be used, including treatment in aqueous or solvent solutions, treatment at cryogenic temperatures (within solvents), and a recently developed method that uses low voltage discharge plasma chemical vapor deposition of osmium tetraoxide (Akahori, Handa et al. 2000). Such methods can be applied as required to enhance conductivity and stabilize specimens prior to embedding within an ICP, and after embedding within an ICP.

Additional conductive stains and cross-linkers

Many methods have been developed to increase the conductivity of biological specimens for TEM. These include metallic "negative" stains such as uranyl and lead salts, reaction of membranes and synthetic polymers with osmium tetraoxide or ruthenium tetraoxide, and sequential protocols with osmium tetraoxide and thiocarbohydrazide, known as the OTO method, which provides sufficient conductivity to examine complex cellular structures with SEM (Allen, Jack et al. 1986; Goldstein, Newbury et al. 1992; Bozzola and Russel 1999), and in conjunction with cryogenic and non-cryogenic methods to image hydrated polymers with TEM and SEM (Goodman, Li et al. 1988; Li, Goodman et al. 1988; Goodman, Simmons et al. 1990). These may also be applied to atom probe analysis. As with the OsO_4 treatment, the heavy metals can also be used to facilitate mass calibration.

Method to Prepare materials into the suitable geometry

In order to use these materials for embedding and stabilizing biological materials it is necessary to form or shape them into the proper geometry. This may be achieved in several ways, each of which will be more suitable for a given type of specimen.

Preparing specimens from cast blocks (*en bloc* methods) will be useful for many specimens, but requires subsequent processing to obtain the necessary configuration for analysis. Figure 4 show the overall protocol and an SEM micrograph of a tip prepared using this method. The particular specimen shown in the micrograph is of ICP embedding media that does not contain protein. Similar specimens have also been prepared with encapsulated proteins. The specimens may be cut as shown by several ways. These include careful trimming by hand with the aid of a very sharp fine point scalpel and a dissecting microscope, by thin section microtomy, and with a micromanipulator apparatus equipped with fine knives specifically designed for this purpose (not shown). The SEM micrograph in Figure 4 shows a tip of osmicated Baytron CPUD2 ICP that has been mounted on the end of stainless steel needle. Such specimens may be imagined in the LEAP and in conventional (non local electrode) atom probes and field ion microscopes. The LEAP, as discussed enables the use of much shorter needles. These may be prepared from ICP embedding materials using broad ion mill etching. An example of such a microtip is shown in Figure 5. Further discussion of this method of specimen preparation follows. Several methods of specimen preparation are summarized in Figures 6-8.

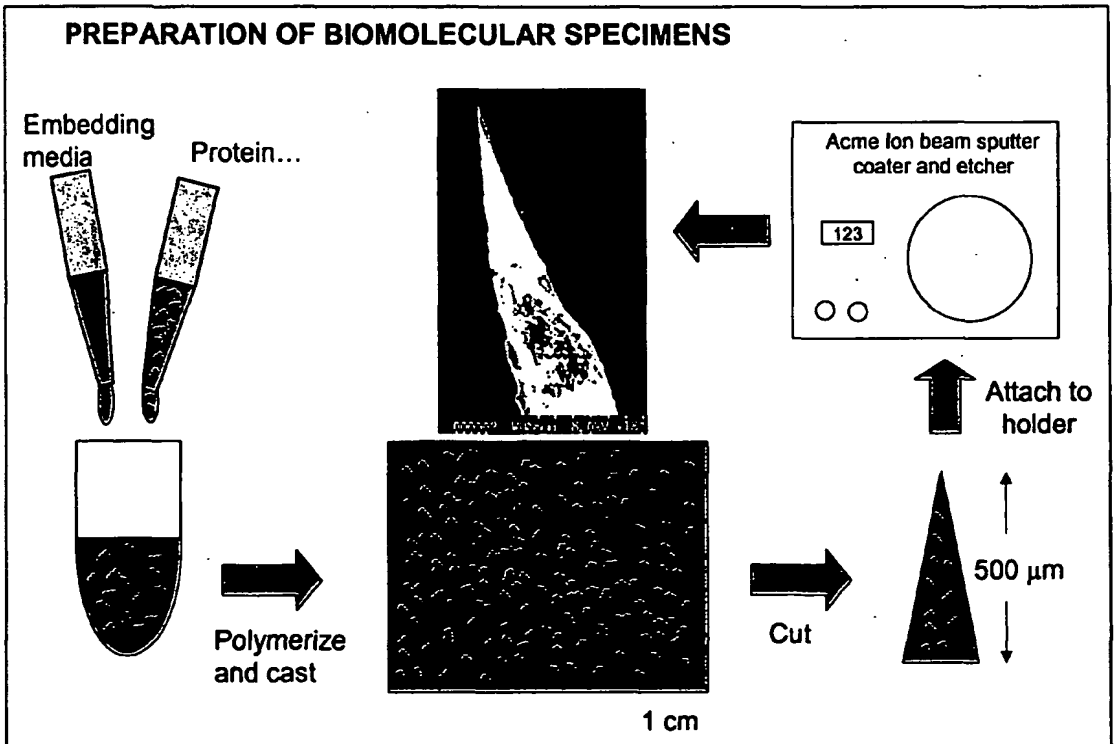

Figure 4: En bloc embedding of a biomolecular specimen. The specimen may be a soluble protein, an insoluble protein, a macro-molecular complex, or a particulate. The specimen is embedded, polymerized or embedded in a supporting matrix of conductive polymer, cut into a micron-scale sharp tip, attached to a holder suitable for either LEAP or conventional atom probe analysis, and then fine sharpened with an argon ion beam.

Specimens in ICP matrix may also be coated onto pre-sharpened tips prepared from metals or conductive silicon (Figure 6 e). As required, any additional sharpening may be readily performed with the FIB tool. Based upon preliminary study, it is generally necessary to use wetting and/or adhesion agents (e.g. epoxy terminated silanes) to improve the bonding stability between the ICP matrix and substrate material. Adhesion promoting agents may be mixed into the ICP, or tips may be pre-coated with a suitable adhesion promoting chemistry (Figure 6 g). The protein or other specimen may also be covalently attached to the tip prior to encapsulation within the ICP (Figure 6 h).

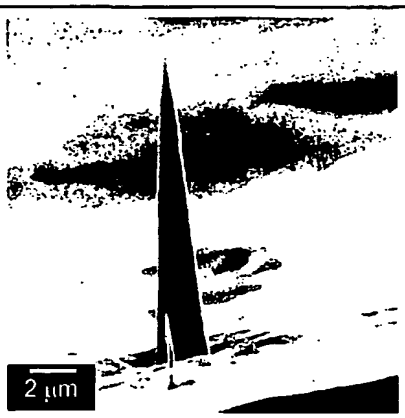

Figure 5: A microtip of bulk CPUD2 ICP prepared with the masking particle and broad ion beam etching on a bulk specimen.

ICPs as *en bloc* embedment matrixes

ICPs may be prepared as monolithic or solid specimens. Monolithic specimens are made by casting or polymerizing specimens within blocks of ICP. For LEAP analysis, the specimen embedded into the ICP matrix is then cut into the proper tip geometry (Figure 6 a-d). This is analogous to *en bloc* embedding for TEM, where the specimen is first embedded and then is subsequently sectioned for TEM imaging. Many separate specimens can be prepared from a single block. Feasibility has been demonstrated by casting Baytron CPUD2, rough shaping with a scalpel, and final sharpening into ~150 nm diameter tip (Figure 8).

There are additional methods to prepare multiple tips on a single specimen block, thereby facilitating high-throughput LEAP analysis (Figure 9). In one method, a focused ion beam instrument (FIB) is used to carve out an annulus to create a buried tip at any desired location on the block. In the second method, masking particles such as diamond dust or metal colloids are placed on the specimen block. The block is then irradiated uniformly with energetic ions at normal incidence to sputter (erode) the surface (Larson, Wissman et al. 2001). At the moment when the masking particles are sputtered to zero mass, multiple tips are left behind that stand clear of the flat surface. This method can be used to simultaneously create dozens of tips on a single specimen block at one time. This method is useful for making multiple tips on a specimen where it is not critical which regions are analyzed. If the masking particles are carefully placed on areas of interest, then specific specimen regions can be selected for LEAP analysis.

Covalent attachment of specimens to tips

Biological and organic specimens such as proteins must be strongly attached to specimen supports (Figures 6g-h, 9). Self-assembled monolayers (SAMS) of organosilanes and thiols are widely used for this purpose in a variety of biotechnology devices with minimal effect on protein function, and by inference, protein structure. SAMS based upon thiols provide very flexible chemistries to link proteins (and ICPs) to gold surfaces. For example, SAMS may be used to link to a variety of functional groups and can be used to pattern protein adsorption (Prime and Whitesides 1991; Mrksich and Whitesides 1996; Ulman 1998). Amino terminal gold-thiol SAMS have been used to link DNA to gold tips for FIM imaging (not shown). Similarly, organosilanes that covalently bond to silicon (and other materials) are available with many

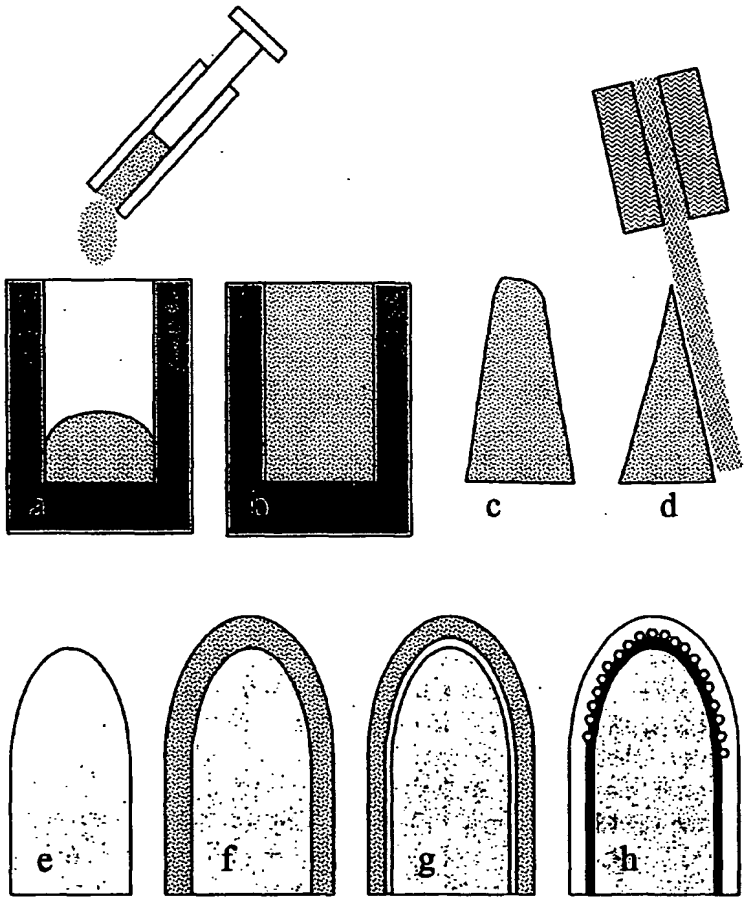

Figure 6: Methods to prepare specimens. Molding specimen-containing ICP embedment *en bloc* (a-b), roughly shaped cast embedment (c), and final sharpening with ion beam (d). Preformed tip fabricated from metal or conductive silicon without (e) and with a thin film layer of specimen-containing ICP embedment (f). A presharpened tip with a self-assembled monolyer (SAM) or other linkage chemistries to enhance bonding of specimen-containing embedment is shown (g), and a similar tip with a layer of specimens bound to a SAM and then coated with ICP embedment (h).

different functional groups to bond to biological specimens (Heiney, Grüneberg et al. 2000; Kumar, Maldarelli et al. 2001) and to adhere to a variety of surfaces including metals and silicon.

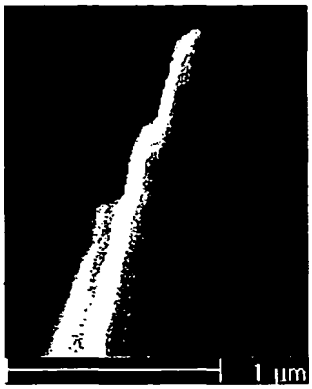

Figure 7: SEM image of a ~150 nm diameter atom probe tip prepared from Baytron CPUD2 polymer.

Cryogenic and non-cryogenic preparation

Cryogenic preparation generally can provide excellent preservation of many specimens, as demonstrated with isolated proteins, proteins within membranes, macromolecular complexes, viruses, and other biological structures (Walther, Chen et al. 1992; Colliver, Brummel et al. 1997; Baker, Olson et al. 1999; Bozzola and Russel 1999; Auer 2000; Baumeister and Steven 2000). Since frozen hydrated specimens have minimal electrical conductivity, it will be necessary to introduce conductivity within the specimen during a freeze substitution step when nanosecond electrical pulsed LEAP is used for analysis. Alternately, all specimen water may be removed during freeze substitution. This is achieved by rapidly freezing (~10^4 or 10^5 °C/second) so that water forms amorphous or vitreous ice, and is most commonly done by plunging the specimen into a cryogen such as liquid ethane cooled by liquid nitrogen (LN_2), or other methods such as high pressure, slam, and propane jet freezing. Specimens are then cryogenically transferred to organic solvents such as ethanol or acetone, fixed, and then embedded at cryogenic temperatures. The specimen may then be prepared at room temperature, or they may be cryogenically sectioned and immuno-labeled or stained at reduced temperatures. Freeze substitution is generally preferred for immuno-histochemistry since it preserves antigenic conformations and enzyme activity (Beesley 1989; Jayasena 1999; White, Sullenger et al. 2000; Shiurba 2001). These functional indicators are strongly suggestive that near native biological structure is maintained. As discussed above, the solvent-based ICP materials are suitable for freeze substitution.

All protocols will require care in order to maintain the highest level of structural preservation. For example, many procedures will require careful dehydration through sieve-dried ethanol for organic solvent based ICP embedments (Panipol F, T and Baytron M+C), and drying from low surface tension solvents (e.g. hexamethyldisilazane or HMDS), use of the critical point method, or freeze drying when preparing materials from aqueous reagents (Albrecht, Rasmussen et al. 1976; Goodman 1999) will be necessary to preserve structure. Direct embedding with aqueous ICPs may also provide a rapid protocol to achieve excellent preservation of hydrated structures.

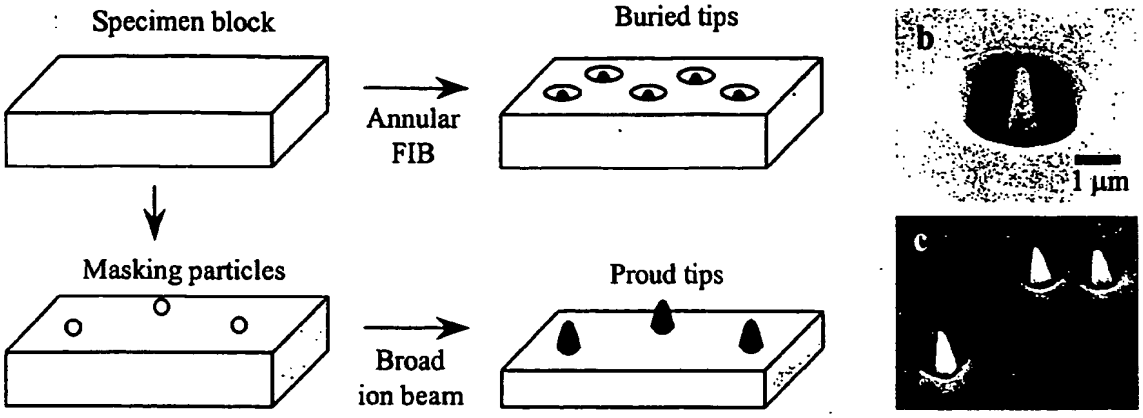

Figure 8: Preparation of LEAP tips from specimens encapsulated in a block of ICP. One method uses a focused ion beam (FIB) to remove a moat around the specimen area of interest (in practice the moat needs to be larger than that shown in figure 8b). The second method applies colloidal particles, diamond dust, or other masking material on the specimen surface. The specimen is then etched or eroded by a wide-field ion beam to produce tips that stand proud of the sputtered surface. The masking particle prevents the erosion of the underlying specimen so that leaves behind a tip that stands proud of the surface. SEM images show tips prepared using FIB annular milling (b) and tips which stand proud of the (sputtered) specimen surface prepared using masking particle etching (c). These tips were prepared in bulk silicon, but may also be prepared with embedding materiasl, as shown in Figure 5 for the masking particle method.

Specimen preparation pathways

Figure 9 diagrams several protocols for specimen preparation. Protein specimens may be encapsulated in a block (*en bloc* method), directly coated onto tips from a blend of specimen in ICP or pre-attached to specimen support tips using linkage chemistries and then coated with ICP (pre-shaped tips). Various chemical agent pretreatments can improve the conformational stability of proteins, such as adding cross-links, and can be used to increase conductivity, such as treatments with osmium tetraoxide, ruthenium tetraoxide, uranyl acetate, glutaraldehyde, tannic acid, and/or potassium permanganate. These treatments may be performed in solution (using centrifugation or filtration rinse steps), or subsequent to attaching the protein to the tip surface. They may also be used to treat bulk specimens such as tissues or cells in culture. The protein or other material is then embedded into the ICP using aqueous prepolymers, solvent castable prepolymers or monomers. These methods can also be applied to non-biological organic and inorganic materials as required.

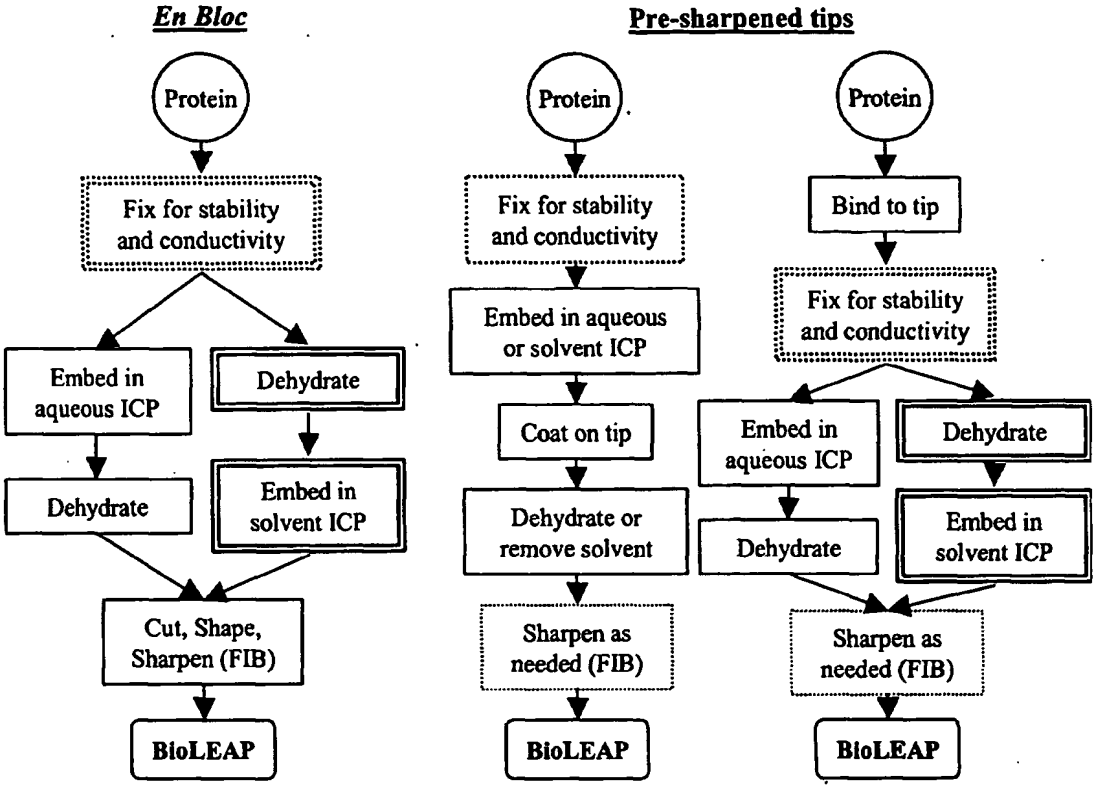

Figure 9: Specimen preparation flow charts. Optional or as needed steps have a dotted outline. Two pathways are illustrated for methods using pre-sharpened tips where: i) the specimen is mixed with the ICP before application to tips, and where ii) the specimen is applied to the tips first, and then encapsulated in ICP. The right-hand pathways with *en bloc* and pre-sharpened tips protocols use solvent castable ICP formulations that are compatible with freeze substitution encapsulation. Steps that may be performed cryogenically are shown with double line borders.

Atom probe analysis of organic materials

As demonstrated above, we have developed and adapted materials with suitable properties to be used as embedments for atom probe analysis of biologicals, organics, particulates and other materials as discussed. These materials have a uniform density as observed in SEM analysis, they can be shaped into the necessary needle like geometry as shown above, they have sufficient electrical conductivity for pulsed electric field LEAP analysis, and other desirable and necessary properties as outlined in Tables 1 and 2.

It is also necessary to demonstrate that it is possible to examine organic materials within the atom probe and obtain mass spectra consisting of small or low molecular weight ions, and to be able to map these ions to specimen locations to create a meaningful and useful 3-D image of the material structure. The following results will demonstrate our current results to achieve this.

The following figures will first illustrate that meaningful mass spectra can be obtained with simple organic model materials including graphite (Figure 10) and pyrolytic carbon (Figure 11). It is very important to note that these simple highly cross-linked "organics" ionized as individual atoms, hence meeting the requirement for high spatial and mass resolution. Since the shape of the tips of these particular specimens did not form into uniform hemispheres, 3-D image reconstruction was not feasible. Figure 10 and 11 nonetheless demonstrate the useful determination of elemental content of carbon-based materials. That 3-D images were not obtained with a particular specimen of an anisotropic materials is not unusual as it is a random process to locate a reasonably uniform specimen area that will generate the necessary hemispherical tip.

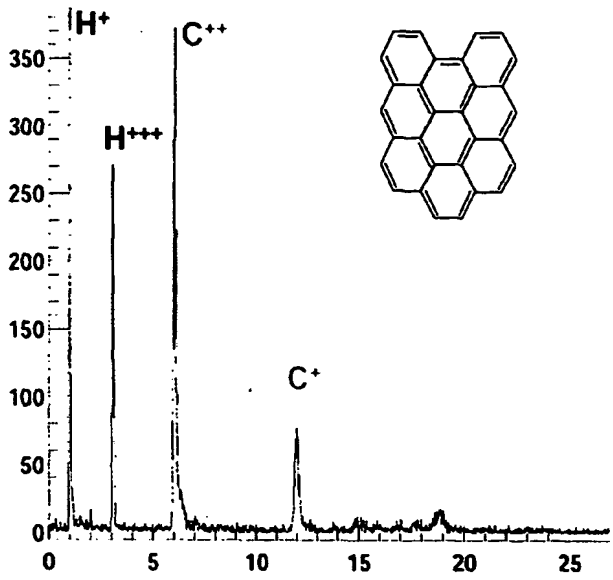

Figure 10: Graphite mass spectra obtained with LEAP. Note low mass fragments that can be clearly identified as single atoms of carbon.

The next figures demonstrate that osmium treated CPUD2 can be imaged with the LEAP. Furthermore, the ions from this material are of low mass/charge, hence of low molecular weight (Figure 12). Since the field strengths are low, we can most likely assume that all ions are in the singly charged state; hence essentially all ions have a molecular weight of less than 100, with almost all less than 50. As discussed above, this is highly desirable in that it enables high spatial resolution and facilitates elemental compositional identity. It should also be noted that previous atom probe analyses of conductive polymers have reported much higher ion masses that are consistent with whole monomers, dimers, and trimers (Maruyama, Hasegawa et al. 1987) that is certainly not desirable for achieving good spatial resolution. The present improved result indicates that the sample preparation methods described herein, including preparation of bulk specimens and osmium treatment, promote formation of low mass ions. In addition, the LEAP microscope provids improvements in performance and localization of the ionization field compared to the instrumentation used in this earlier study.

While Figure 12 shows the mass spectra, it is difficult to assign elemental composition to each peak due to limited mass resolution with the particular LEAP instrument used for this analysis. This is because many different elements or combinations of elements within a molecular fragment can have quite similar masses that are not fully resolvable. Secondly, the ionization

behavior of organic materials within the LEAP is not yet well characterized. It is anticipated that further study with a higher mass resolution LEAP in conjunction with the analysis of different materials of known composition will facilitate the assignment of elemental and molecular composition to different observed mass/charge ratios. Figure 13 is a view of a 3-D reconstruction of the ionized fragments of the same specimen. This illustrates that each mass fragment can be localized to a different 3-D location and thus suggests that reconstruction of the 3-D structure is feasible.

LEAP ANALYSIS OF PYROLYTIC CARBON HEART VALVE

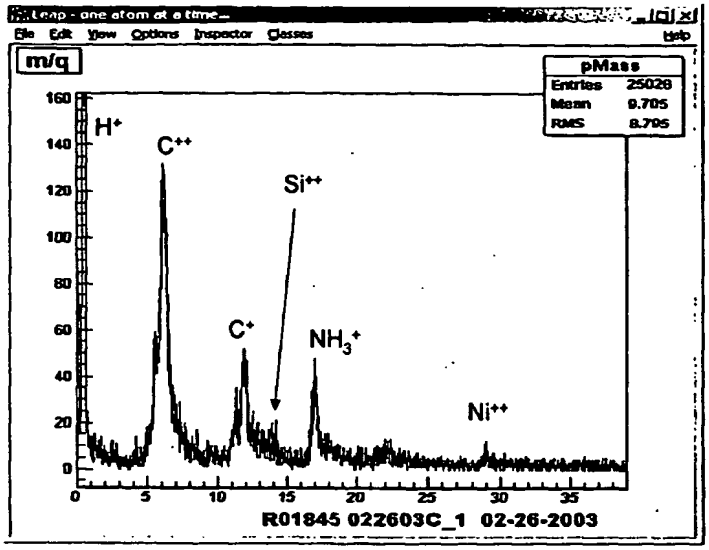

Figure 11: Mass spectra from pyrolytic carbon that was manufactured for use a heart valve prosthetic. Note the low mass/charge fragments. This material also contains some silicon and nitrogen, as has been previously determined and disclosed. The nitrogen appears to ionize as NH_3, based upon its mass. A nickel coating was applied to the specimen prior to sharpening the material with a focused ion beam instrument.

The embedding material based on Baytron CPUD2 consists of 3 separate polymers, which include a poly(pyrrole), a poly(styrenesulfonate), a poly(urethane) and some additional minor components. Although firm identification of the various ions has not yet been made, the 3-D reconstructed ion image appears to indicate that ionized low mass fragments and elements from each of these components are in separate areas, hence supporting the ability of the LEAP to image these structures. For example, certain mass fragments that are tentatively identified as the styrene and sulfonate groups constituents of poly(styrenesulfonate), appear in close proximity as they should be since the sulfonate is a pendant group on the styrene (Figure 14). Similarly, several lower mass ions including elemental carbon and carbonyls also appear localized in close proximity, which is consistent with image reconstruction of the polyurethane component. Since polyurethanes have substantial chemical similarity to proteins, this suggests that these materials and the processes described herein will enable the 3-D composition and structural analysis of proteins with the LEAP microscope.

Imaging considerations

Imaging proteins within embedding materials will require identifying the protein within the embedment by virtue of it differing elemental composition and structure. This is possible since the LEAP identifies all elements with atomic-scale resolution. Since the linear structure of both the embedment polymer(s) and most generally the protein are known, this can be used to

facilitate this analysis. For example, the sulfur atom in the polythiophene monomer will enable ready separation of the matrix from the embedded protein since sulfur is rare in proteins. In the case of proteins, the regular repeating backbone of carbon-carbon-nitrogen can also be used to highlight the protein structure. Other specimens may be readily identified by similar elemental features, such as the phosphorus in DNA and RNA. In addition, identification may be further simplified using heavy metal labels or isotopes (including deuterium) incorporated into either the protein or the ICP embedding matrix.

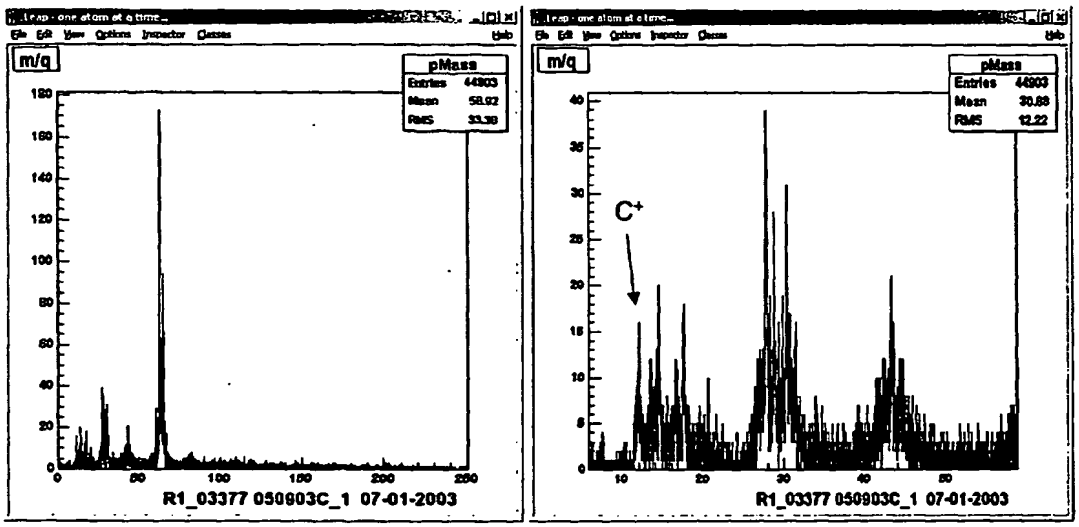

Figure 12: LEAP mass spectra of osmium treated CPUD2. The large peak in the full mass width image appears to be Cu, and not part of the CPUD2 itself. Copper is used to form the specimen support and may be present in the sample due to indirect sputtering that occurred during the ion etching sharpening process.

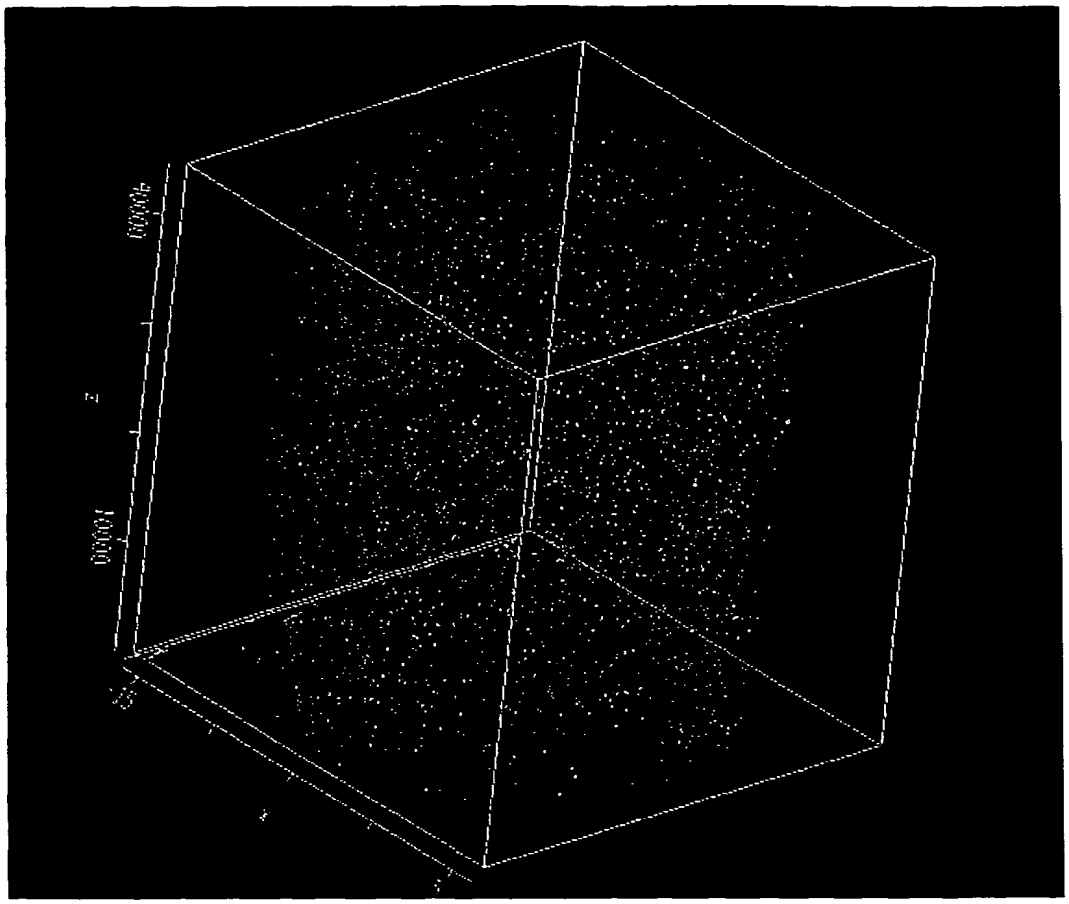

Figure 13: LEAP 3-D image reconstruction of osmium treated CPUD2. Most masses with clear peaks are shown. The larger dots represent the larger masses, while small dots represent smaller masses. The mass peak that may be Cu is not shown.

LEAP Software and informatics

The LEAP intrinsically determines where ions come from and what their mass/charge ratio is. From the mass/charge ratio it is possible to calculate the elemental composition of an ion, given adequate mass resolution and potentially aided by some knowledge of the material being examined. However, with respect to organics and biomolecules, understanding the connectivity of the specimen atoms (and/or molecular fragments) is what is desired. Since the LEAP or other atom probes do not directly provide this information, computational methods are required for providing this information and for its visualization. It should be noted that the position sensitive ion detector in the LEAP detects only about 60% of specimen ions. Therefore, within any given field of view, up to 40% of atoms (or molecular ions) within the specimen are not known. Some desirable features for computational analysis include:

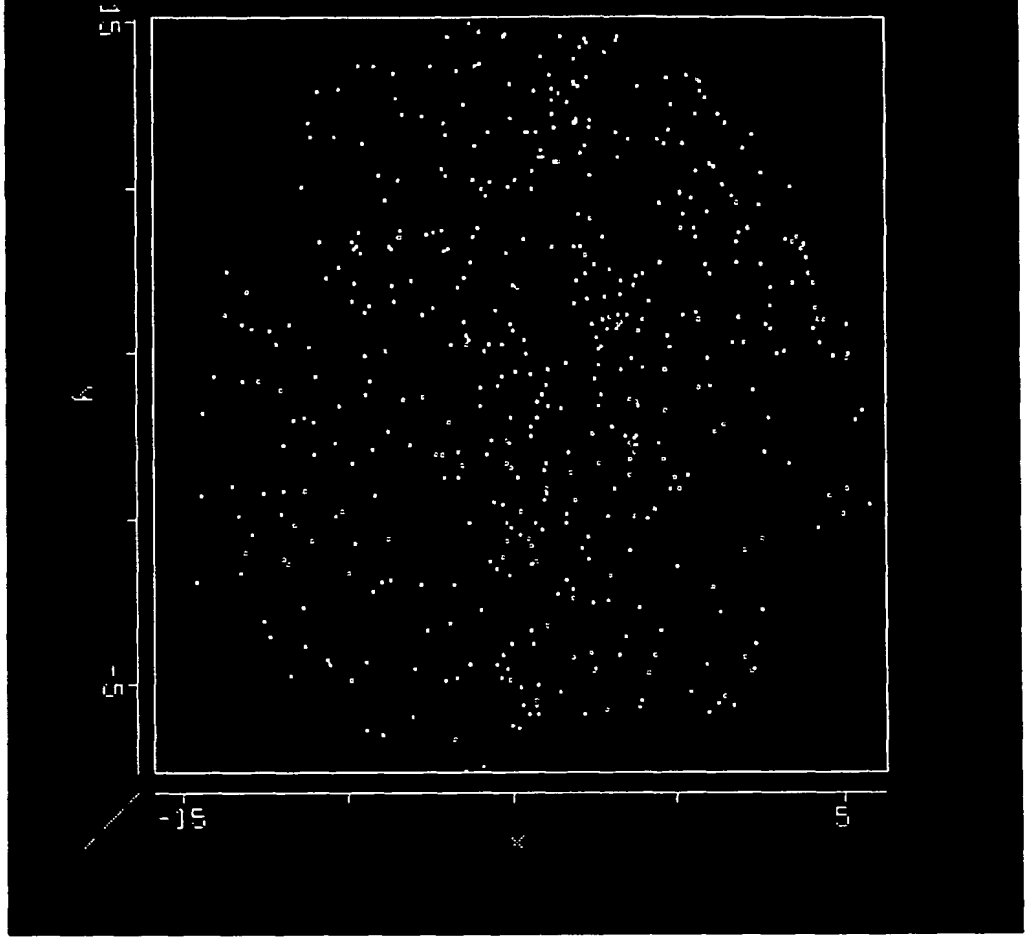

Figure 14: A small slice through the same 3-D reconstruction shown in Figure 13. Only the two large masses are shown, and that may correspond to the styrene and the sulfonate groups of the poly(styrene sulfonate) component of Baytron CPUD2.

- Algorithms to locate amino acid carbon-nitrogen "backbones" in proteins.
- Algorithms to locate the ICP matrix so that it may be subtracted from the image to find the protein.
- Best fit matching of LEAP data to known bond lengths and angles.
- Best fit matching of LEAP data to sequence information (when available) in order to enable calculations of missing atoms.
- Algorithms to utilize data from other sources such as homology modeling, NMR, crystallography, and mass spectrometry.
- Algorithms to average several structures and/or piece together multiple incomplete structures of proteins. Incomplete structures may be observed at the boundaries of an atom probe

sample, and if an individual protein collapses or is entirely ionized at some time during BioLEAP analysis.

- Software to determine the likely elemental composition of small molecular ion fragments.

The overall approach for the analysis software is illustrated in Figure 15.

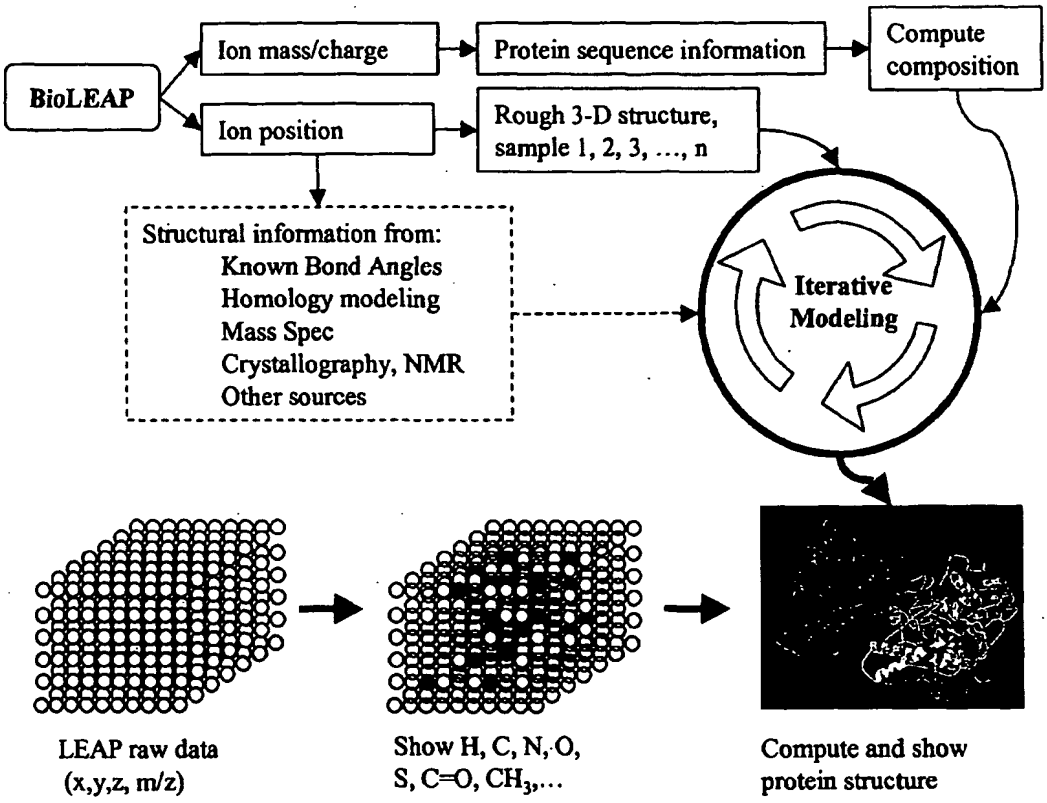

Figure 15: Software protocol for the analysis of proteins with the BioLEAP. The upper block diagram illustrates the data processing pathway. The lower figures graphically illustrate the process of extracting the protein structure from the background ICP matrix, followed by computation and display of the protein structure in the desired format (illustrated as a ribbon format).

Summary

This document has described the need for preparing biological, organic and several other types of materials for analysis with 3-D atom probe microscopy. Subsequently, several methods and new uses of matter were discussed and demonstrated to enable such analysis. These included materials and methods for preserving biological and organic structure, methods and materials to embed specimens, and methods and apparatus to prepare specimens into the proper geometry

for analysis with LEAP microscopes and other atom probe microscopes. Finally, the analysis of LEAP images and compositional analysis were presented and discussed, as were the types of computational analysis that are required to make the most value of atom probe images. These results demonstrate that the methods, materials and processes described herein can enable the 3-D compositional and structural analysis of many biological, organic, and other materials that heretofore could not be analyzed with atomic and near atomic resolution.

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